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Original Article

Establishment of rat pneumococcal meningitis models: a histopathological analysis

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Abstract: The aim of this study was to perform a preliminary investigation of the pathogenesis of bacterial meningitis-induced brain injury by establishing rat pneumococcal meningitis models. Infant Wistar rats were intracranially inoculated with different concentrations of *Streptococcus pneumoniae*. Rats were sacrificed at different time points to observe clinical symptoms and pathological changes in brain tissues. Twenty-four hours after intracranial inoculation with *Streptococcus pneumoniae*, regardless of high or low concentrations of bacterial inoculation, all rats developed bacterial meningitis with manifestations such as lethargy and seizures. Pathological changes in brain tissues included subarachnoid and intraventricular inflammation, vasodilation and vascular congestion, and cortical neuronal necrosis. The number of rats with seizures, the degree of cerebral vascular disease, and the extent of neuronal damage were associated with the concentration of bacterial inoculum. Thirty days after infection, brain tissue weight significantly reduced. The pathological changes induced by inoculation with pneumococcal meningitis in Wistar rats were similar to those seen in the human brain. The possible mechanisms of brain damage caused by meningitis are cerebrovascular inflammation and disruption of regional cerebral blood flow.

Keywords: Streptococcus pneumonia, meningitis, animal model, pathology

Introduction

Bacterial meningitis is a common infectious disease of the central nervous system in developing countries [1]. The incidence of bacterial meningitis in young infants remains unchanged since the 1980s and is associated with significant mortality [2]. Bacterial meningitis is more common in the neonatal period than at any other time in life [3]; this can cause significant brain damage, resulting in permanent neurological sequelae. Invasive bacterial meningitis [4], which is a rare but deadly neurological emergency [5], continues to be a substantial cause of morbidity and mortality [6] and the prognosis for infants is particularly poor [7]. Recently, with the improvement of living conditions in developing countries [8], the availability of comprehensive primary vaccinations (PCV7, PCV13 and PPSV23) [9], and the use of antibiotics supplemented by hormone therapy [6], the prognosis for bacterial meningitis has greatly improved. However, the mortality rate is still high, and neurological sequelae such as stroke are common. Studies have shown that antithrombotic therapy may become necessary during the treatment of bacterial meningitis, as it has two preventive effects for stroke [10]. *Streptococcus pneumoniae* meningitis is one of the most common forms of bacterial meningitis, and *Streptococcus pneumoniae*, which was the most common bacterial meningitis pathogen in the United States during 1997 and 2010 [6], is a major pathogen leading to neurological sequelae. This study aims to establish an animal model of bacterial meningitis, which accurately replicates infection conditions for the further study of disease pathogenesis, treatment, and prevention of complications.

Materials and methods

Preparation of the bacterial suspension [11]

Three strains of *Streptococcus pneumoniae* (No. 31003, provided by Chinese Pharmaceu-

tical and Biological Products, Beijing Institution, China) were seeded on sheep blood agar medium (Beijing Obo Star Biotechnology Co. Beijing, China) and grown overnight at 37°C with 5% carbon dioxide, then inoculated in broth. Bacteria were collected when grown to mid-logarithmic phase, centrifuged and washed with saline, centrifuged again, and diluted with saline to a concentration of $3\times10^{4\text{-}6}$ colony forming units (cfu)/mL.

Experimental animals

Seventy-two Wistar rats aged 21 days (provided by Shandong University Animal Center) were randomly allocated to the experimental group (n=64) or the control group (n=8). The experimental group was divided into six subgroups depending on time of sacrifice: 24 h, 48 h, 72 h, 10 days, 20 days, and 30 days. The 24 h group was further subdivided into three groups based on the concentration of bacterial inoculum: 3×10^4 cfu/mL, 3×10^5 cfu/mL, and 3×10^6 cfu/mL, with eight rats per group. The concentration of bacterial inoculum for the 48 h and 72 h groups and the 10-, 20-, and 30-day groups were all 3×10^4 cfu/mL. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University.

Inoculation in rats

After anesthesia by intraperitoneal injection of pentobarbital sodium (40 mg/kg) (Hubei Ding Fu Chemical Co., Ltd., Hubei, China), meningitis was induced using the method of Kim et al [12]: a cerebellar cistern puncture was performed, 20-40 μL of CSF was removed, and 50 μL of *Streptococcus pneumoniae* bacterial suspension was injected at different concentrations. Rats in the control group were injected with 50 μL saline. Twenty-four hours after the bacterial inoculation, rats of each test group were given ceftriaxone (100 mg/kg, qd, intraperitoneal injection) for 7 days.

Clinical observation

The inoculated rats were housed in cages at room temperature with free access to food. Twenty-four hours after inoculation, clinical manifestations score was calculated as follows [12]: 0 points: normal walking activities; 1 point: slight reduction in walking and activity; 2 points: slow to turn upright; 3 points: cannot turn upright and have an increased number of seizures.

CSF examination

Twenty-four hours after bacterial inoculation, rats were anesthetized. CSF was obtained from the cerebellomedullary cistern, cultured, and colonies were counted.

Weight detection

Rats in each group were weighed at the end of the predetermined experimental time. Rats were then killed using the cervical vertebra fracture method, the skull was opened to extract the brain, and brain weight was recorded.

Histological examination

At the end of the predetermined experimental time, rats were anesthetized, and left ventricular perfusion was performed using 4% paraformaldehyde to fix body tissues. Brains were fixed overnight in 4% paraformaldehyde and embedded in paraffin the following day. The brains were then sectioned along the coronal place into 5-μm slices for HE staining. Inflammation of the meninges and brain tissue was observed, and the semi-quantitative counts for a vascular disease index (0=no abnormal blood vessels; 1=1-3 abnormal blood vessels/slice; 2=4-8 abnormal blood vessels/slice; 3≥8 abnormal blood vessels/slice) and a neuronal damage index (0=normal; 1=1-2 lesioned areas/each brain hemisphere; 2=3-4 lesioned areas/each brain hemisphere; 3≥4 lesioned areas or integration into a large lesioned area/each brain hemisphere; 4≥50% hemisphere involvement) were performed using a 200× light microscope [12]. Six slices were used from each specimen, and 10 random images from each slice were used for observation.

Statistical analysis methods

Data are expressed as mean ± sd. Analysis of variance was used to perform comparisons among multiple groups. Comparisons among multiple sets of semi-quantitative data were carried out using the Wilcoxon signed-rank test.

Table 1. Inoculated bacteria concentration and clinical parameters

Compared item	3×10^4 group (n=8)	3×10^5 group (n=8)	3×10^6 group (n=8)	Statistics	P
Bacterial titers in CSF (cfu/ml)	$(1.5\pm0.9)\times10^2$	$(1.8\pm0.5)\times10^2$	$(1.6\pm0.7)\times10^2$	2.95	>0.05
Symptom score	2.0±0.7	1.8±0.9	2.5±0.2	2.415	>0.05
Incidence of seizures	0	1 (12%)	3 (37.5%)	10.82	<0.05
Vascular lesion index	1.0±1.1	1.8±1.2[a]	2.3±1.0[a,b]		<0.01
Neuron damage index	0.8±1.2	1.6±1.4[a]	2.5±0.5[a,b]		<0.01

Note: Compared with the 3×10^4 group, [a]$P<0.01$; Compared with the 3×10^5 group, [b]$P<0.01$.

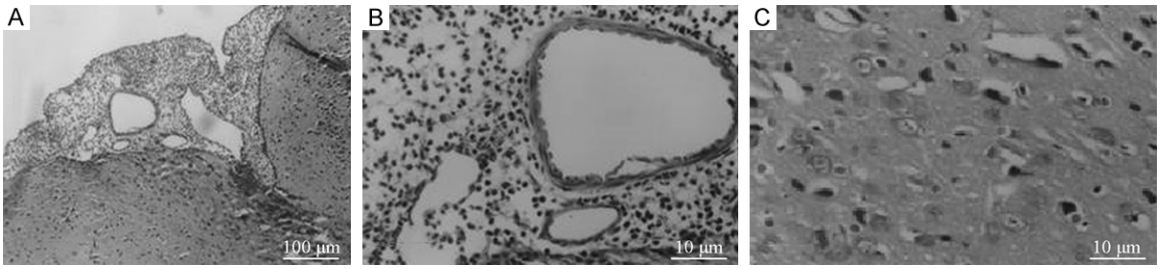

Figure 1. Cerebral HE staining for the test group (A: Subarachnoid; B: Meninges; C: Cerebral cortex).

The Chi-square (χ^2) test was used to compare rates among groups. The relationship between rat neuronal injuries, vascular disease index, and concentration of inoculated bacteria was analyzed by Spearman rank correlation analysis.

Results

Clinical observation

Twenty-four hours after bacterial inoculation, all rats developed bacterial meningitis regardless of bacterial inoculation concentration. The rats with fewer symptoms showed somewhat less motion and decreased feeding, but those with more symptoms showed severe poor mental status, poor feeding, lethargy, and lags in response time, ataxia, and convulsions. Symptom scores between the different bacteria concentrations had no significant differences (H=2.415, $P>0.05$). However, there was a significant difference in the number of seizures between rats in the different bacteria concentration groups ($\chi^2=10.82$, $P<0.01$), and there was a high incidence of seizures in the high bacteria concentration group (**Table 1**). Rats in the control group showed normal activity and good feeding behavior, without any of the symptoms mentioned above. The meningitis symptoms of rats treated with antibiotics had resolved within seven days, but about 10% of the rats had motor dysfunction sequelae.

CSF examination

CSF from all rats was cultured and inoculated with the same strain of *Streptococcus pneumoniae* with a higher bacterial titer. The bacterial titer of CSF for rats inoculated with different concentrations of bacteria had no significant differences (F=2.95, $P>0.05$) (**Table 1**). The CSF of rats in the control group had no bacterial growth.

Brain weight

Thirty days after infection, the brain weight of the experimental group was 1.38±0.18 g, which was significantly lower than the brain weight of the control group (1.65±0.12 g), (t=5.485, $P<0.05$). At 24 h, 48 h, 72 h, 10 days, and 20 days after inoculation, however, the experimental group had no significant differences in brain weight compared with the control group. Body weight at each time point showed no significant differences between the two groups.

Histopathology

Twenty-four hours after bacterial inoculation, visual observation of brains in the experimental group revealed edema, and the brain parenchyma had minor bleeding. The brains of rats inoculated with the higher-concentration (3×10^6 cfu/mL) bacterial suspension had more obvi-

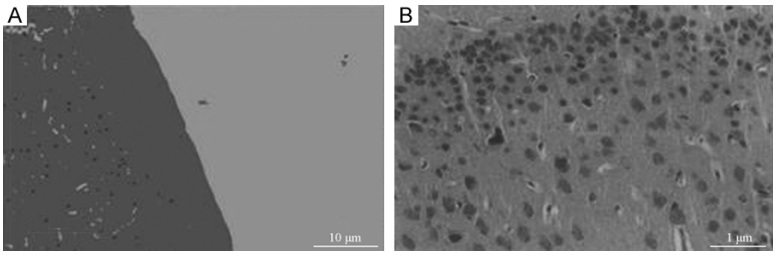

Figure 2. Cerebral HE staining for the control group (A: Meninges; B: The cerebral cortex).

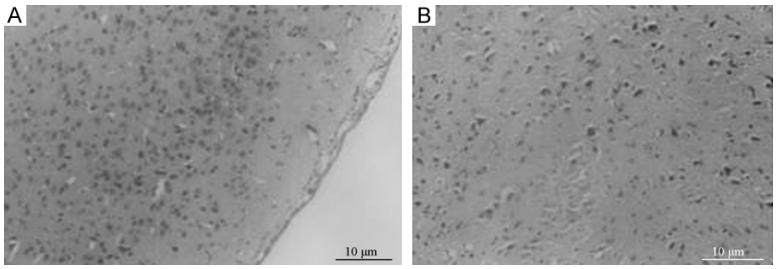

Figure 3. Rat cerebral cortex HE staining of the test groups treated by bacterial inoculation with different concentrations (A: Low concentration; B: High concentrations).

ous changes. Observations using light microscopy revealed neutrophils, macrophages, and fibrinous exudates in the subarachnoid space, ventricles, and pia mater (**Figure 1A**). Subarachnoid blood vessels and brain parenchyma showed edema, vasodilatation, and hyperemia (**Figure 1B**). Neural cells were swollen and deformed, and there was evidence of glial proliferation (**Figure 1C**). No meningeal inflammation or neuronal damage was observed in the control group (**Figure 2**). Bacterial inoculum concentrations were positively correlated with the pathological vascular index (r^2=0.77, P<0.01) (**Table 1**). The neuronal damage index was also associated with the concentration of bacterial inoculum (r^2=0.685, P<0.01) (**Table 1**). The area of neuronal loss was smaller when the concentrations of bacterial inoculum were lower (**Figure 3A**); larger necrotic lesions were visible within the brain parenchyma when the concentrations of bacterial inoculum were higher (**Figure 3B**).

In rats inoculated with bacteria for 72 h, subarachnoid inflammation was still present, but the significant changes at this point were swollen neurons in the inner granular layer and occasional neutrophil infiltration within monocytes of the brain parenchyma (**Figure 4A**). Dentate gyrus granule cell damage was visible in the hippocampus close to the ventricles (**Figure 4B**), but no significant changes were seen in CA1 and CA3 neurons although they are known to be sensitive to hypoxia. Hippocampal neurons in the control group had no injury (**Figure 4C**). No basal ganglia neuronal necrosis was observed.

After antibiotic treatment, 10 days after inoculation, the density of some cortical neurons was increased. The arrangement of neurons was haphazard, and there was evidence of glial cell proliferation.

Discussion

Currently, animal experimental models are the basis for the study of human diseases. Stable animal models established by simulating disease characteristics in humans can help clinicians to explore disease pathogenesis, find effective prevention measures, and fight human diseases [13]. Due to acute disease onset and atypical clinical symptoms, treatment for bacterial meningitis is often delayed. This delay can threaten children's lives, and some patients have subsequent epilepsy, mental damage, and other sequelae. Therefore, establishing reliable animal models of bacterial meningitis is very important for the exploration of factors causing brain injuries and for determination of effective treatment programs [14]. In this study, neuropathological changes associated with human bacterial meningitis in infants were stimulated by establishing animal models of meningitis through bacterial inoculation.

In this experimental model, a bacterial suspension was injected into the cisterna magna of rats to cause meningitis. Previous meningitis models were induced by systemic infection through intravenous or intraperitoneal injection of bacteria, which may more accurately reflect the manifestation and development of human meningitis. However, the success rate of meningitis models induced by systemic infection

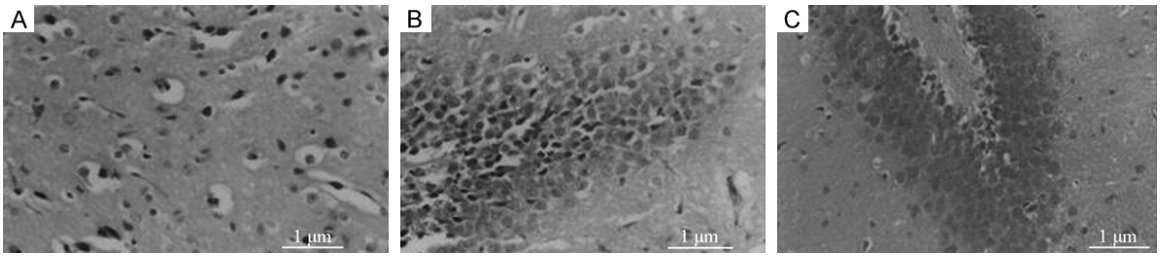

Figure 4. Rat brain in 72 hours after inoculation of bacteria (A: The brain parenchyma in the test group; B: Hippocampus in the test group; C: Hippocampus in the group).

routes was low. The positive rate of bacteria in rat CSF culture was less than 50%, and the meningitis symptoms of the rats with positive CSF bacteria were mild with no brain damage; further, the rats had a high mortality rate due to systemic infections involving multiple organs [15]. The meningitis model using intracranial inoculation of bacteria was not particularly time-consuming, and meningitis can be induced in 24 h. Kim found that neutrophils appeared in the cavum subarachnoidale within 4 h of injecting streptococci B into the cisterna magna of neonatal rats [12].

In our experiments, pathological changes in the central nervous system in rats with pneumococcal meningitis were similar to those seen in humans [16]: both showed subarachnoid and intraventricular inflammatory infiltration, vascular dilation, congestion and cortical neuronal necrosis. Although perfusion was performed for all brain specimens, blood vessels still showed vascular congestion. This indicates that the congested blood vessels did not open before vascular perfusion, suggesting that systolic and diastolic dysfunction occurred because of blood vessel wall inflammation [12]. However, the characteristics of vascular disease had certain differences between human and animal models of meningitis. Human meningitis is characterized by inflammation infiltrating the vessel wall, lasting for several days, whereas the vascular pathological changes of intracranial meningitis for animals were acute and transient, characterized by significant expansion and congestion of blood vessels [12]. The mechanisms causing this difference are still unclear, but may be related to route of infection and disease duration.

Pathological changes relating to neuronal injury may provide a method for exploring the pathogenesis of meningitis brain injury. Kim found that when rats had bacterial meningitis, neuronal loss regions were consistent with the vascular lesion regions, and cortical ischemic injury-like changes (wedge necrosis) occurred. It is believed that intracranial hypoperfusion caused by bacterial meningitis in rats may be one of the reasons for cerebral cortex injury [12], because in human meningitis there is a hemispheric reduction in perfusion [17]. The role of hypoperfusion in rat meningitis was not very clear, and it was found that changes in brain blood flow could cause whole brain ischemia and hypoxia [18]. In our experimental animals, however, hippocampal CA1 and CA3 neurons which are typically highly sensitive to ischemia were not damaged, suggesting that ischemia due to brain meningitis in rats was not fully hemisphere ischemic brain, which was different from cerebral ischemia damage alone [12].

Different concentrations of bacteria were injected in order to observe the resulting effect on degree of brain damage, which will contribute to the study of different degrees of meningitis. The study found that with the injection of different concentrations of bacteria, CSF bacterial titers of rats in each group had no significant differences, but there were significant differences in the extent of damaged blood vessels and neurons. The high concentration of bacteria caused more significant vascular and neuronal damage, suggesting that low concentrations of bacteria required a longer time to reach saturation titer after injection. When higher concentrations of bacteria were injected, the bacterial titers in the CSF needed a relatively short period to reach saturation, so the time of the brain exposure to the saturated bacterial titer was longer resulting in more severe brain injury [19]. When rats were treated with an intraperitoneal injection of antibiotics following an infection of more than 24 h, the antibiotics cured the meningitis and allowed the rats to

survive. This provides convenient conditions for observing and studying the pathogenesis mechanism and prevention measures for the bacterial meningitis sequelae [20].

In summary, following a direct infusion of different concentrations of bacteria to the CSF, higher bacterial inoculum concentrations were associated with more severe cerebral cortex damage in the rat. The symptoms of bacterial meningitis progressively improved with prolonged antibiotic treatment time, which provides some guidance regarding follow-up clinical treatment. However, because the time using antibiotics in this study was short, further studies are needed to determine the full range of benefits provided by extending treatment time.

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Disclosure of conflict of interest

None.

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